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Chromatin Boundaries in Budding Yeast: The Nuclear Pore Connection

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Summary

Chromatin boundary activities (BAs) were identified in *Saccharomyces cerevisiae* by genetic screening. Such BAs bound to sites flanking a reporter gene establish a nonsilenced domain within the silent mating-type locus *HML*. Interestingly, various proteins involved in nuclear-cytoplasmic traffic, such as exportins Cse1p, Mex67p, and Los1p, exhibit a robust BA. Genetic studies, immunolocalization, live imaging, and chromatin immunoprecipitation experiments show that these transport proteins block spreading of heterochromatin by physical tethering of the *HML* locus to the Nup2p receptor of the nuclear pore complex. Genetic deletion of *NUP2* abolishes the BA of all transport proteins, while direct targeting of Nup2p to the bracketing DNA elements restores activity. The data demonstrate that physical tethering of genomic loci to the NPC can dramatically alter their epigenetic activity.

Introduction

Various structural and biochemical studies of the cell nucleus presage the existence of tethers that somehow impose its organization. One important notion supporting nuclear tethers is the confinement of chromosomes into relatively discrete nuclear territories and biochemical/structural studies that provide evidence for chromatin loops (reviewed in Cremer and Cremer, 2001; Hart and Laemmli, 1998). Despite a wealth of structural and biochemical information, mechanisms whereby nuclear order is brought about and dynamically altered remain enigmatic. We have undertaken a genetic-functional approach to address this mystery, based on the assumption that the activity of the chromatin boundary/insulator elements may mechanistically be linked to nuclear structure.

Boundary elements are specialized DNA sequences that operationally partition the genome into functional domains (reviewed in Gerasimova and Corces, 2001; West et al., 2002). When flanked by these elements, genes are protected from the repressive or activating effects of nearby heterochromatin or enhancer elements, respectively. Experimentally, this protection is characterized by a position-independent expression of transgenes. When interposed between an enhancer and a promoter, such elements (not necessarily all) can also insulate a gene toward activation by enhancer elements.

At the biochemical level, several proteins implicated in boundary functions have been characterized. Examples of such boundary proteins are the vertebrate protein CTCF (Bell et al., 1999) and the two *Drosophila* proteins, suppressor of Hairy-wing [Su(Hw)] (Geyer and Corces, 1992; Holdridge and Dorsett, 1991) and BEAF-32 (Hart et al., 1997; Zhao et al., 1995). This latter protein binds with high affinity to the *scs′* DNA region, which defines one of the boundary elements of the *Drosophila* 87A7 hsp70 heat shock locus (Zhao et al., 1995). The other partner element of this domain, *scs*, binds a protein called Zw5 (Gaszner et al., 1999). The *scs′* and *scs* elements were initially identified and characterized by pioneering work led by Schedl's laboratory and shown to mediate boundary and insulating activity both in flies and in tissue culture cells (Kellum and Schedl, 1991; Udvardy et al., 1985; Zhao et al., 1995).

While much progress has been made concerning the role of boundary elements in development, genomic imprinting, and gene expression, little is known about their mechanism of action. Various boundary models have been discussed. Some are based on local or more long-range changes in chromatin structure, and others suggest that boundary function may be brought about by nuclear compartmentalization (reviewed in Gerasimova and Corces, 2001; West et al., 2002). Interesting recent experimental evidence supports a possible link between nuclear order and chromatin boundaries (Gerasimova et al., 2000).

To screen genetically for boundary activities, we constructed a yeast "boundary-trap" strain (K. Ishii and U.K. Laemmli, submitted). For this approach, two selectable genes were inserted into the silenced mating-type locus *HML* of budding yeast, a locus that shares molecular features with metazoan heterochromatin such that inserted genes are epigenetically repressed (Loo and Rine, 1995). Synthetic DNA binding sites for chimeric proteins flank one of the inserted genes. This boundary-trap strain adopts different expression states depending on whether genuine boundary proteins that block spreading of heterochromatin or transcription activators are targeted to its *cis*-acting elements (K. Ishii and U.K. Laemmli, submitted). Here, we present a genetic screen obtained with a targeted yeast genomic library. Interestingly, a class of boundary activities was identified that appear to mediate their epigenetic function by specific physical tethering to the nuclear pore complex (NPC).

Results

A Boundary-Trap Reporter for Genetic Screening

A boundary-trap reporter was engineered that can distinguish between transcriptional activators (TAs) and chromatin boundary activities (BAs, defined below) that operate by mechanisms distinct from TAs. An unequivocal distinction between these activities is important since it is well known that targeting of TAs proximally

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and upstream of promoters can readily activate silenced genes (Aparicio and Gottschling, 1994; Sekinger and Gross, 2001).

The boundary-trap reporter KIY54 was found to be suitable for this purpose (K. Ishii and U.K. Laemmli, submitted). Briefly, it contains two selectable markers (*ADE2* and *URA3*) inserted between the E and I silencers of the *HML* locus (Figure 1A). At this heterochromatic locus, both genes are silenced due to the Sir protein complexes, which spread from the E and I silencer elements along the chromatin fiber (Loo and Rine, 1995). The *ADE2* reporter was flanked by UASg elements that interact specifically with the DNA binding domain of Gal4 (Gbd).

Reporter strain KIY54 adopts three main expression states as cartooned in Figure 1A. If its UASg elements are vacant or occupied by a neutral/inactive protein, then most cells will harbor silenced *ADE2* and *URA3* genes (OFF/OFF state). A genuine BA is defined as a function that protects *ADE2* from silencing without activation of *URA3*, thereby creating an ON/OFF expression state. In contrast, if the UASg sites are bound by TAs, both genes are expressed (ON/ON state). These expression states of *ADE2* and *URA3* are conveniently determined by cell growth under selective conditions. The diagnostic plate for the ON/OFF state produced by BAs lacks adenine but contains FOA (5′-fluoroorotic acid), which kills cells containing the *URA3* gene product (Boeke et al., 1984). Importantly, TAs or TA/BA hybrid proteins do not yield colonies on this diagnostic plate (see below) due to *URA3* expression. Plates lacking adenine score for the number of cells that are either in the ON/ON or ON/OFF expression states, and plates containing FOA scores for the number of cells that are in OFF/OFF or ON/OFF states (Figure 1A).

The experimental potential of KIY54 is illustrated by demonstrating the evolutionarily conserved BA of the *Drosophila* BEAF protein (Figure 1B). If KIY54 expresses no relevant protein (None) or the Gal4 DNA binding domain (Gbd) only, virtually no cells (less than 1 in 2×10^5) are in an ON/OFF state. This is experimentally manifested by a lack of growth on the double-selection plate (−Ade/+FOA), even at the lowest dilution. In contrast, robust growth is observed on the double-selection plate if the C-terminal domain (aa 203–283) of *Drosophila* BEAF is expressed (Gbd-C^{BEAF}) (Figure 1B; K. Ishii and U.K. Laemmli, submitted). Quantitative titration revealed that this BEAF construct protects 15%–25% of the cells from silencing *ADE2* while maintaining *URA3* repressed.

For the sake of illustration, we included data obtained by expression of the middle domain (aa 82–202, M) of BEAF, which encompasses a feeble TA function amounting to about 3% of that of Gbd-VP16 (K. Ishii and U.K. Laemmli, submitted). We observed that the M domain alone or linked to the C domain (constructs Gbd-M^{BEAF} and Gbd-MC^{BEAF}, respectively) behaved as a TA by activating both *ADE2* and *URA3* (ON/ON state) in KIY54 (Figure 1B). Thus, the TA function of the M domain in KIY54 is experimentally dominant over the BA function of the C domain. This observation and further studies with many other TAs demonstrated that KIY54 identifies BAs but scores efficiently against TAs or hybrid proteins (K. Ishii and U.K. Laemmli, submitted).

Above considerations identify KIY54 as a powerful boundary-trap tool suitable for genetic screening. Toward this end, KIY54 was transformed with a Gbd-fused yeast genomic library and scored for colonies that grow on double-selection plates. Many of the plasmids recovered from such colonies express genuine BAs that could be grouped into different functional classes. One interesting class, discussed here, mechanically links chromatin boundaries to nuclear structure.

Various Proteins Involved in Nuclear Transport Harbor Robust Boundary Activity

Several proteins with a strong BA were identified by genetic screening that are involved in the transport of macromolecules between the nucleus and the cytoplasm (termed transportins here, reviewed in Gorlich and Kutay, 1999). The BA function of these transportins is experimentally manifested by robust growth of KIY54 on the double-selection plate (−Ade/+FOA) of Figure 1C. Quantitative titration determined that about 10%–30% of the cells are in an ON/OFF epigenetic state (*ADE2* protected from silencing) if these transportin are expressed (indicated in Figure 1C); these values are comparable to that of Gbd-C^{BEAF}. Note that the values obtained by titration for the portion of cells in the different epigenetic states can significantly vary if different transformed colonies are tested.

Most of the transportins belong to the importin β-like protein superfamily (Figure 1C). These well-studied proteins, represented by exportins and importins, are known to form complexes with Ran/GTP and different cargoes for directional transport through the nuclear pore complex (NPC) (reviewed in Gorlich and Kutay, 1999; Mattaj and Englmeier, 1998). Cse1p, the yeast counterpart of mammalian CAS, is an exportin dedicated to the transport of importin α (Srp1p) back into the cytoplasm (Hood and Silver, 1998). The other exportin, Los1p, obtained in the screen (mammalian homolog exportin-t) exports tRNA from the nucleus (Hellmuth et al., 1998). Mex67p (mammalian homolog TAP) doesn't belong to the importin β superfamily but is also involved in RNA export, particularly for mRNA (Segref et al., 1997). Sxm1p is a typical importin that functions in the nuclear import of Lhp1p, a protein implicated in the maturation of tRNA (Rosenblum et al., 1997). The final isolated protein, Gsp2p, is one of the two Ran homologs (98% identical to Gsp1p) of yeast, which play central roles in determining the directionality of the nuclear/cytoplasmic transport (Belhumeur et al., 1993).

The Boundary Function of Cse1p and Los1p Map to Defined C-Terminal Domains

Clearly, the boundary function of the various transportins was unexpected. Is this observation a nonspecific artifact or reflective of a novel interaction? As a possibility, we considered that these BAs might arise from a perturbed protein import/export situation. But the observation that expression of $Cse1^{474–960}$ lacking Gbd exhibited no BA (Figure 2B) argues against this interference and shows that DNA tethering to the UASg sites is necessary. Moreover, it seems very unlikely that the RNA exportins Los1p and Mex67p could have a similar indirect effect as Cse1p. That is, it is difficult to conceive that expression of these functionally different

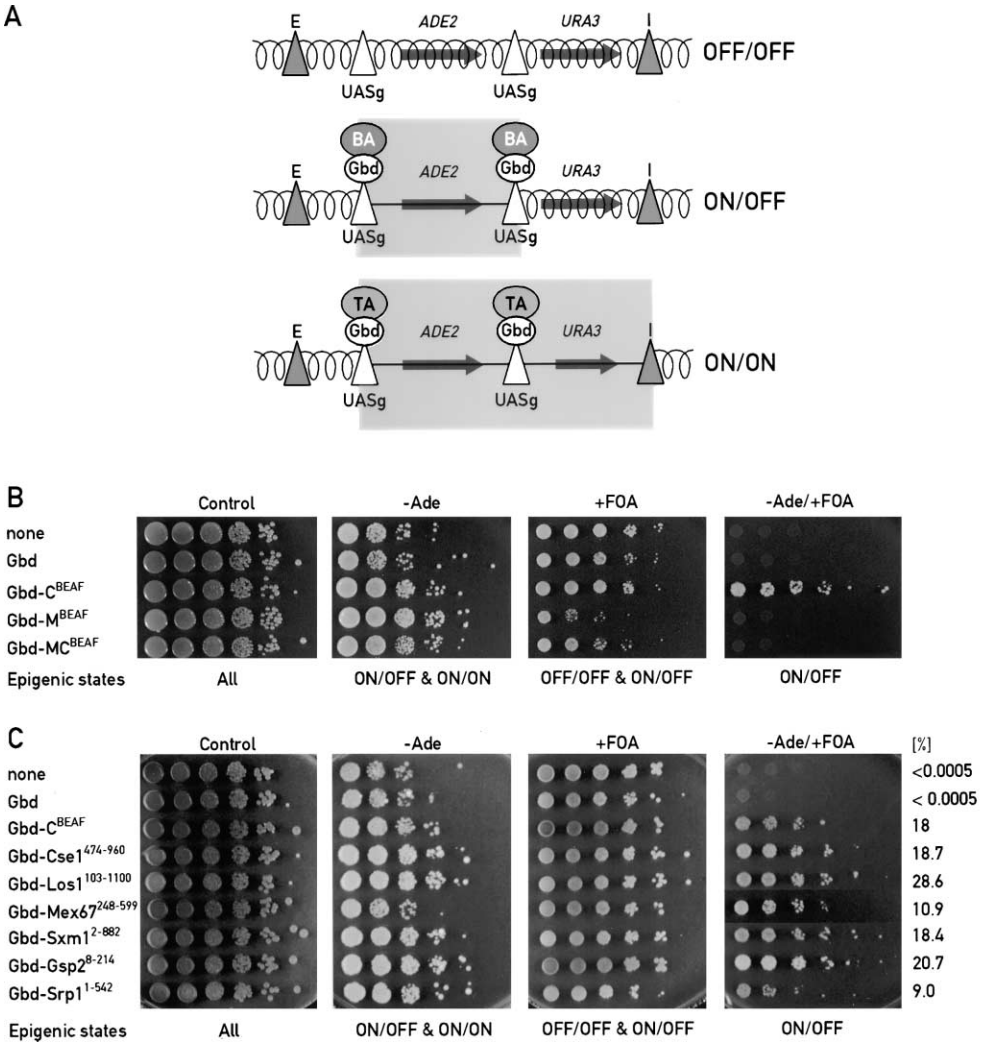

Figure 1. Diagnostic Epigenetic State for Genuine Boundary Activities: Identification of Transportins

(A) This cartoon shows the three main epigenetic expression states of KIY54. This strain harbors the *ADE2* and *URA3* genes between the E and I silencers of the *HML* locus. The *ADE2* gene is flanked by four UASg sites, which specifically bind the Gal4 DNA binding (Gbd) domain. If the UASg sites are empty or occupied by a neutral protein, *ADE2* and *URA3* are predominantly inactive (OFF/OFF), indicated by coils (top). If the UASg sites are occupied by a genuine boundary protein, which blocks spreading of heterochromatin and lacks transcription activation potential, an ON/OFF epigenetic state is predominantly established. That is, *ADE2* is stochastically protected (=ON) from silencing (depicted by a line), while *URA3* remains silenced as it remains exposed to the I silencer (middle). In contrast, transcription activators or hybrid proteins, which harbor both a boundary and a transcription activation potential, mediate an *ADE2*/*URA3* = ON/ON state (bottom). The different epigenetic states of KIY54 allow an unequivocal distinction between genuine boundary proteins that block heterochromatin and transcription activators.

(B) The experimental potential of KIY54 is shown by spotting 10-fold serial dilutions of KIY54 cells on four different agar plates. The different Gbd-fusion proteins tested are indicated on the left. The epigenetic states assayed are indicated at the bottom, and the plates are labeled at the top. All epigenetic states grow on the nonselective "control" plate, which scores for the applied cell concentration. The plate called −Ade (lacks adenine) measures the fraction of cells that are either *ADE2*/*URA*3 = ON/ON or ON/OFF. The plate called +FOA measures the fraction of cells harboring a silenced *URA3* gene, which are either ON/OFF or OFF/OFF. The double-selection plate labeled −Ade/+FOA identifies cells expressing genuine boundary proteins that protect *ADE2* from silencing while maintaining *URA3* silence (ON/OFF).

(C) The boundary activity of the various transportins obtained by genetic screening was tested in KIY54 as described in (B). The different Gbd-fusion proteins used are listed on the left where superscripted numbers reflect the amino acid stretch tested. The activity of full-length Srp1p was also shown. The percentage of cells in the ON/OFF state as determined by quantitative titration is indicated (left). Note that these values can significantly vary if different colonies are compared.

exportins establishes a physiological state that somehow specifically protects *ADE2* from silencing.

Is the BA mediated by sheer "molecular bulk," where spreading of heterochromatin is nonspecifically blocked by the size of the transport complexes? The export complex of Cse1p, for example, includes Srp1p (importin α) and Ran/GTP (Gsp1p or Gsp2p). Possibly supporting a molecular bulk scenario is the finding that any member of this complex, fused to the Gbd domain for targeting, can mediate the BA (Figure 1C). Hence, it suffices to target the Cse1p export complex to the UASg to block epigenetic silencing of *ADE2*.

But do the transport complexes need to be intact? We proceeded to address this question by experimentally

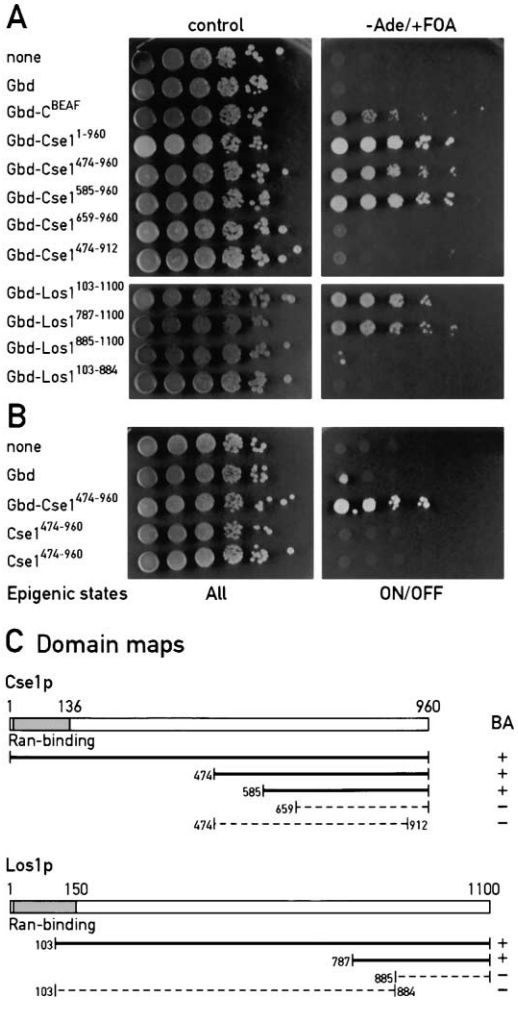

Figure 2. The BA of Exportin Cse1p and Los1p Maps to Well-Defined Domains

(A) Various N- and C-terminal deletion constructs were prepared from Cse1p and Los1p and their BA tested in KIY54. Some representative constructs are shown. Note that the BA of both proteins maps to well-defined C-terminal domains and that the N-terminal domains containing a putative Ran binding domain are dispensable.

(B) Various Cse1p constructs were tested in KIY54. Note that targeting Cse1p to the UASg sites with the help of Gbd is required to obtain a BA.

(C) The domain maps of Cse1p and Los1p are shown. Constructs represented by thick lines harbor a BA; those represented by dashed lines are inactive.

defining the protein domains bearing the BA. The data for Cse1p demonstrate that its BA is localized to the C-terminal domain, defined by construct Gbd-Cse1$^{585–960}$ (Figure 2A). Importantly, this activity is sharply lost if either 185 or 48 amino acids are additionally deleted either at the N- or C-terminal end in constructs Gbd-Cse1$^{659–960}$ and Gbd-Cse1$^{474–912}$, respectively. Deletion studies with Los1p also map its BA to a small C-terminal domain of 314 amino acids (Gbd-Los1$^{787-1100}$) (Figure 2A).

Cse1p and Los1p belong to the importin β superfamily, which shares putative sequence motifs for Ran/GTP binding at their N-terminal domain (Figure 2C; Gorlich et al., 1997). Since formation of the export complex requires interaction with Ran/GTP (Mattaj and Englmeier, 1998), it is unlikely that the small C-terminal domains of Cse1p and Los1p, which bear the BA, support formation of the bulky export complex. These observations argue against a molecular bulk model to account for the selective loss of a heterochromatin structure between the *cis*-acting UASg sites. Moreover, if this model were to apply, then genetic screening should have yielded other unrelated protein complexes bearing a BA. What then is the common molecular mechanism for the BA of this family?

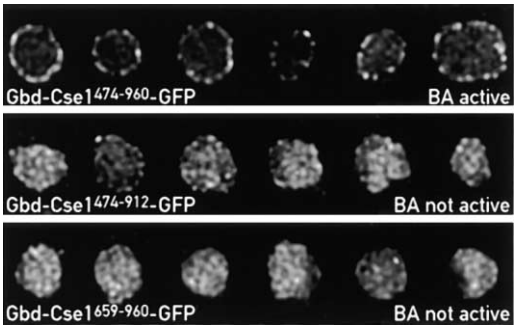

Figure 3. The Boundary Function of Cse1p Requires Interaction with the Nuclear Rim

The cellular position of Cse1p C-terminal fragments tagged with GFP was determined by fluorescence microcopy. Note that the construct Gbd-Cse1$^{474–960}$-GFP, which harbors a boundary activity (BA active), is predominantly bound to the nuclear rim. In contrast, deletion derivatives (Gbd-Cse1$^{474–912}$-GFP and Gbd-Cse1$^{659–960}$-GFP), which lack activity (BA inactive), localize to the nuclear lumen. Note that these GFP constructs were expressed in strain KIY54 whose UASg sites at *HML* must also bind these proteins. The low abundance of these binding sites (eight sites), however, precludes that this interaction is optically detected from the bulk location of the Cse1p constructs.

The Boundary Function of Cse1p Requires Interaction with the Nuclear Rim

To approach an understanding of the BA mechanism, we focused our attention on Cse1p. Previous immunofluorescence and GFP localization studies demonstrated that Cse1p is principally associated with the nuclear rim (Hood et al., 2000). Is a peripheral localization of Cse1p essential for its BA? To answer this question, GFP fusion proteins were constructed whereby this tag was C-terminally fused to yield constructs Gbd-Cse1$^{474–960}$-GFP, Gbd-Cse1$^{659–960}$-GFP, and Gbd-Cse1$^{474–912}$-GFP. Note that these GFP-tagged proteins behaved similarly in the boundary assay as their untagged versions (data not shown).

Micrographs of yeast cells revealed a tight experimental correlation between association with the nuclear rim and boundary activity (Figure 3). This figure shows that the C-terminal domain of Cse1p, construct Gbd-Cse1$^{474–960}$-GFP, which harbors the complete BA, also mediates association with the nuclear rim (top row). In contrast, the deletion versions of this fragment that lack any BA, constructs Gbd-Cse1$^{659–960}$-GFP and Gbd-Cse1$^{474–912}$-GFP, are no longer associated with the nuclear rim. In these cases, the GFP signal is predominantly found throughout the nucleus (bottom two rows). Note that these GFP

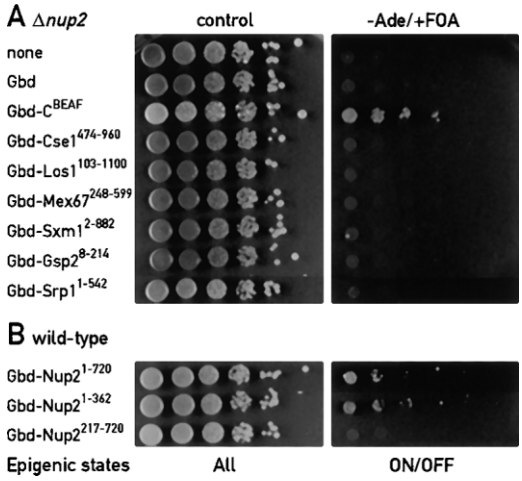

Figure 4. The Boundary Activity of the Transportins Requires Nup2p

(A) Nup2p predominantly localizes to the inner nuclear basket of the NPC to which certain export (and also import) complexes dock during nuclear-cytoplasmic traffic. This figure shows that the BA of all transportins, but not that of BEAF, is abolished in a KIY54 derivative whose *NUP2* gene was deleted (Δ*nup2*).

(B) Demonstrates that full-length Gbd-Nup2p constructs and N-terminal derivatives harbor BA when tested in KIY54. See Figure 1B for experimental details and nomenclature.

constructs were expressed in strain KIY54 whose UASg sites at *HML* must bind these proteins. But the low abundance of these binding sites (eight sites flank *ADE2*) precludes that this interaction was separately detected from the bulk location of the Cse1p constructs.

The experiments above indicate that the BA of the transportins may require association with the nuclear rim.

The Boundary Activity of the Exportins Requires *NUP2*

The various transport complexes are known to dock on the nuclear side to the so-called basket of the nuclear pore complex (NPC). This docking step is particularly well studied for the Cse1p export complex, and recent evidence suggested that Nup2p, which is predominantly associated with the NPC basket, serves as the initial receptor for this export complex (Booth et al., 1999; Hood et al., 2000). This notion is based on biochemical interaction studies and mislocalization of Cse1p in a Δ*nup2* strain wherein Cse1p was no longer at the nuclear rim, but predominantly found throughout the nucleoplasm. Strains with a deleted *NUP2* gene are known to be viable, supposedly since it has overlapping functions with two other nucleoporin genes, *NUP1* and *NSP1* (Loeb et al., 1993).

Since Cse1p is mislocalized in a Δ*nup2* strain, it was of interest to test the BA of the transportins in this genetic background. The spot test series presented in Figure 4A show that the BA of Gbd-Cse1$^{474–960}$ was abolished in a Δ*nup2* strain (no growth on the double-selection plate). In contrast, the BA of the C-terminal domain of BEAF (Gbd-C^{BEAF}) was similar to that observed in wild-type cells. The BA of the other transportins (Gbd-Los1$^{103–1100}$, Gbd-Mex67$^{248–599}$, Gbd-Sxm1$^{2–882}$, Gbd-Srp1$^{1–542}$, Gbd-Gsp2$^{8–214}$) was also completely lost in a Δ*nup2* strain.

A concern about the finding above is that the loss of boundary function in Δ*nup2* might be indirect, perhaps caused by a perturbed transport despite normal cell growth. To address this question, we asked whether targeting Nup2p directly to the UASg of the *HML* reporter could establish a BA directly in the wild-type KIY54 strain. Indeed, the data show that full-length Nup2p mediates good blocking activity (Gbd-Nup2$^{1–720}$) (Figure 4B). The N-terminal fragment of Nup2p (aa 1–175) is known to be necessary and sufficient for Nup2p association with the NPC basket (Hood et al., 2000) and for the genetic complementation of strains harboring mutations either in *NSP1* or *NUP1* (Loeb et al., 1993). The observation that the smallest deletion construct harboring BA (Gbd-Nup2$^{1–362}$) encompasses this essential fragment of Nup2p (aa 1–175) suggests that the BA of Nup2p may arise from NPC tethering. In addition, in vitro interaction experiments have established that the domain of Srp1p, which carries the BA, corresponds to the domain that mediates the interaction with Nup2p (data not shown).

By contrast, the BA of BEAF, unlike that of the transportins, is Nup2p independent, indicating that different mechanisms are involved. The data further suggest that transportins may protect *ADE2* from silencing by tethering its flanking UASg elements to the NPC basket. This tethering can be executed indirectly by any member of the Cse1p export complex (Cse1p, Gsp2p, Srp1p), the importin and exportins (Sxm1p, Mex67p, and Los1p), or alternatively can be achieved by direct association with Nup2p.

Specific, Physical Tethering of Genomic Sites to the Inner NPC Basket

The experiments above beg the question of whether it is possible to physically tether different genomic loci, such as *HML*, to the inner NPC basket. We addressed this question by immunolocalization, live imaging, and chromatin immunoprecipitation. Previous studies showed that genomic loci could be visualized if they are tagged with an array of Lac operator (LacO) sequences and the GFP-LacI protein is expressed (reviewed in Belmont, 2001). We asked whether it was possible to tether such foci to the NPC as mediated by Cse1p. Toward this end, the C-terminal domain of Cse1p was inserted into the middle of GFP-LacI to yield the triple domain protein, GFP-Cse1$^{474–960}$-LacI.

The GFP proteins were expressed in two different strains, one carrying a LacO array near the late origin of replication ARS1413 (chromosome XIV) and the other near the early origin of replication ARS908 (chromosome IV). Spheroplasted cells derived from these strains were immunostained with the monoclonal antibody MAb414 (Davis and Blobel, 1986) to visualize the NPC (red) and an anti-GFP antibody to highlight the genomic loci (green). Figure 5 shows representative samples of these cells. This panel shows that the LacO foci of both strains (ARS1413 and ARS908) are generally away from the red NPCs and embedded in the blue nucleoplasm (DAPI, top and third row). In contrast, we observed that these LacO foci generally (over 95%) colocalize with or directly abut the red NPC signal in either strain expressing GFP-Cse1$^{474–960}$-LacI (second and fourth rows).

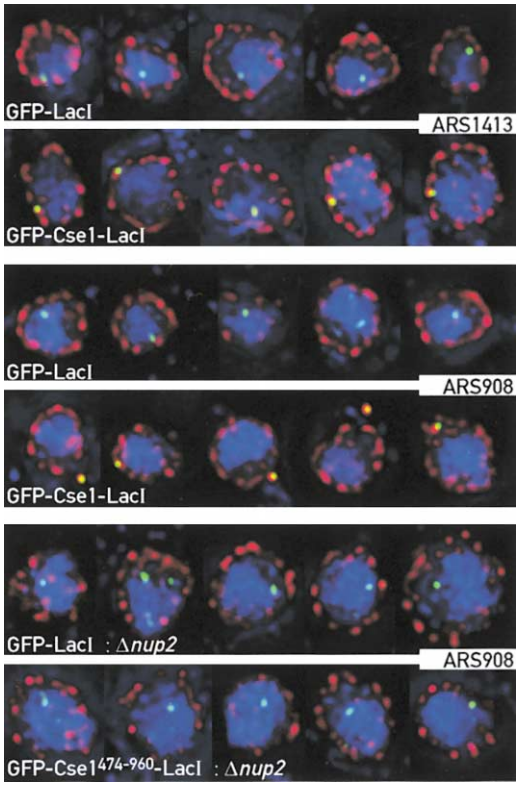

Figure 5. Physical Tethering of Genomic Loci to the Inner NPC Basket

Exponentially growing yeast cells were fixed and immunostained for the NPC (red, MAb414) and GFP (green). DNA was highlighted with DAPI (blue). Note that MAb414 is known to recognize a variety of NPC component (Davis and Blobel, 1986). Two yeast strains were used that harbor LacO arrays near the late or early replications origins ARS1413 (chromosome XIV) and ARS908 (chromosome IV), respectively. These strains expressed either construct GFP-LacI or GFP-Cse1$^{474-960}$-LacI as indicated. We observed that expression of GFP-Cse1$^{474-960}$-LacI (not GFP-LacI) mediated tethering of the LacO foci to the NPC. These GFP constructs were also expressed in a tagged ARS908 strain whose *NUP2* gene was deleted (Δ*nup2*). In this background, tethering by GFP-Cse1$^{474-960}$-LacI of the LacO foci to the NPC was not observed.

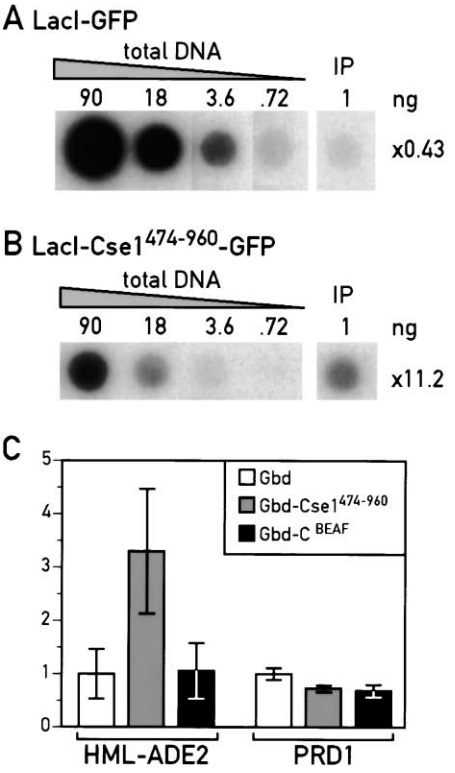

Figure 6. Tethering of the LacO Array and of the *HML* Locus to the NPC

Chromatin immunoprecipitation with the NPC antibody MAb414 using the LacO-tagged ARS908 strain or CLY1. (A) and (B) show the DNA hybridization signal probed with LacO DNA to 1 ng of IP DNA derived from the ARS908 strain compared to four dilutions of total DNA (90, 18, 3.6, and 0.72 ng) as indicated. Relative enrichment is indicated to the right. Note that the number of LacO repeats in the strain expressing GFP-Cse1$^{474-960}$-LacI is reduced relative to the one expressing GFP-LacI.

(C) The bar graph shows the relative enrichment of *ADE2* DNA (0.5 kb from second UASg) or of control *PRD1* DNA (10 kb from *HML*) in the IP DNA fractions derived from CLY1 cells expressing one of the constructs indicated. DNA samples were quantitatively assessed by real-time PCR. The values obtained were normalized to that obtained with the strain expressing Gbd. Note the Cse1p-dependent enrichment of *ADE2* DNA (*HML-ADE2*) in the IP fraction obtained with MAb414.

We demonstrated above that the boundary function of transportins is lost in Δ*nup2* strains (Figure 4), while previous studies established that NPC association of Cse1p requires Nup2p (Hood et al., 2000). In line with expectation, we observed that the Cse1-mediated association of the LacO foci with the NPC is lost in the Δ*nup2* background (Figure 5, bottom two rows).

These observations establish that the GFP foci move from the nuclear lumen to the nuclear rim if the C-terminal domain of Cse1p is inserted into the GFP-LacI protein. This relocalization is lost in Δ*nup2* strains. Importantly, the Cse1p-mediated interaction yields a single focus and not ring-like signals. This suggests that the LacO DNA array (not only the chimeric proteins) is NPC bound, since foci arise by repetitive binding of the GFP fusion protein to this array. Without this interaction, a ring-like signal would be expected, as was obtained with construct Gbd-Cse1$^{474-960}$-GFP, which contains the Gal4 instead of the LacI DNA binding domain (Figure 3).

Chromatin immunoprecipitation (ChIP) experiments directly established the Cse1p-mediated NPC association of the LacO arrays. Chromatin obtained from cells expressing either GFP-LacI or GFP-Cse1$^{474-960}$-LacI was immunoprecipitated with the NPC-specific monoclonal antibody (MAb414). The DNA fractions obtained were then dot-blotted and hybridized to LacO sequences. Quantitative assessment of the data revealed a significant enrichment (about 11-fold) of the LacO sequences in the immunoprecipitated fractions derived from cells expressing GFP-Cse1$^{474-960}$-LacI relative to those obtained from GFP-LacI-expressing cells (Figures 6A and 6B).

We extended this tethering question directly to the *HML* reporter of KIY54 using ChIP. The resulting fractions were quantitatively assayed for DNA sequence content by real-time PCR. We observed that *ADE2* DNA

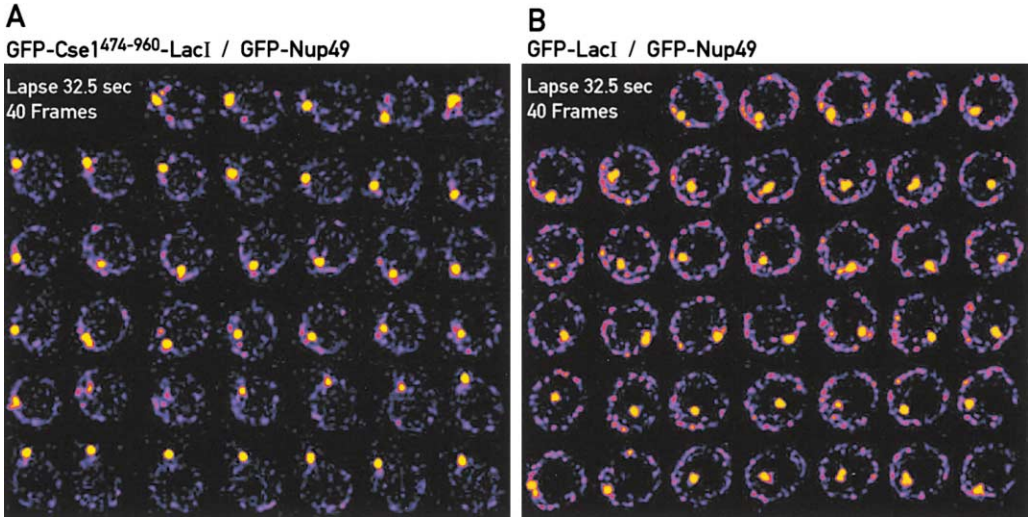

Figure 7. Live Imaging of NPC-Anchored LacO Foci

(A) Live imaging of cells that express GFP-Cse1$^{474-960}$-LacI and harbor a LacO array near ARS1413. Frames (40) were recorded every 32.5 s and are shown in false color. Note that the LacO foci remain predominantly bound to the NPC, which was visualized by expression of GFP-Nup49p. The LacO focus appears to visit the nucleoplasm in the frames shown in the third row and possibly visits the cytoplasm in the frames shown in the last row.

(B) As in (A) but cells expressing GFP-LacI. Note that the LacO foci remain predominantly in the nuclear lumen but ricochet often off the nuclear periphery.

fragment near the UASg site (0.5 kb) is enriched about 3.3-fold in the MAb414 IP fraction derived from cells expressing Cse1p$^{474–960}$ relative to that of cells expressing Gbd or Gbd-C^{BEAF} (Figure 6C). As an additional control, we examined the association of *PRD1* (10 kb from *HML*) and observed no relative enrichment for this locus in the IP fraction (Figure 6C).

Live Imaging of a NPC-Tethered Genomic Locus

Time-lapse imaging of live cells nicely corroborated the immunofluorescence and biochemical data (Figure 7). The LacO focus of cells expressing either construct GFP-Cse1$^{474–960}$-LacI or the control construct GFP-LacI were recorded for different periods of time. The nuclear rim was visualized by GFP-tagging the NPC gene *NUP49*. Note that both LacI and *NUP49* were tagged with GFP. But since the former is ring-like and the latter is seen as a bright dot (both shown in Figure 7 in false color), it is easy to distinguish them visually. Figure 7A shows an example of a live cell expressing GFP-Cse1$^{474–960}$-LacI recorded every 32.5 s over a 13 min time period (40 frames). An identical time-lapse recording of a cell expressing GFP-LacI is shown in Figure 7B. Examination of Figure 7A shows that the LacO dot of this cell expressing GFP-Cse1$^{474–960}$-LacI is generally tethered to the nuclear rim with only a single visit to the nuclear interior (row 4). Curiously, we noted in such time-lapse movies that the LacO foci of such cells appear sometimes at the exterior of the nuclear rim (bottom row, Figure 7A). This observation raises the intriguing question of whether a chromatin section can visit the cytoplasm. Figure 7B shows that the LacO dot is generally nucleoplasmic in cells expressing GFP-LacI, although frequent transient interactions with the nuclear rim can also be observed. Note that Gasser's laboratory has studied in a different experimental context the dynamic behavior of LacO foci in cells expressing GFP-LacI in some detail (Heun et al., 2001).

The experiments above establish several key points: live imaging demonstrates that the C-terminal domain of Cse1p mediates the Nup2-dependent physical tethering of two genomic LacO arrays to the NPC; and ChIP data extend tethering notion at the DNA level to the LacO array of ARS908 and, importantly, also to the *HML* locus of strain KIY54.

These observations support the view that the BA of Cse1p and, by extension, that of the other Nup2p-dependent transportins, arises by specific physical anchoring to the NPC.

Discussion

We aimed to genetically identify boundary activities (BAs) that block the propagation of heterochromatin by molecular mechanisms other than transcription activation, with the hope of identifying novel nuclear functions that affect gene expression. This identified a hitherto unknown player of epigenetics; the nuclear pore complex (NPC). Interestingly, previous theoretical speculations have assigned different structural and functional roles to the NPC in the biology of the nucleus (Blobel, 1985).

Genetic screening for such boundary activities conspicuously identified a family of proteins (transportins) implicated in the transport of various cargoes between the nucleus and the cytoplasm. Undoubtedly, a striking and unexpected finding was that different members of the transportin family could mediate boundary function. Nonetheless, a congruent series of genetic experiments, in vitro and in vivo localization, and biochemical studies

are nicely consistent with a single molecular mechanism. Namely, that transportins block the propagation of heterochromatin by direct or indirect tethering of the *cis*-acting boundary elements to the Nup2p receptor of the NPC basket.

Diagnostic Epigenetic States for Boundary Proteins

The genetic screen was carried out with the boundary-trap strain KIY54, whose powerful screening potential is based on its low background and its differential epigenetic expression states, depending on whether its UASg sites are occupied by BAs or TAs (Figure 1). To approach mechanistic questions about boundary function, we consider it crucial to know whether a protein overcomes silencing by a BA (heterochromatin blocking) or a TA. While in the former case a molecularly ill-defined phenomenon is studied, in the latter case, a simple molecular competition between silencing and "nonsilencing" factors may occur. Moreover, we argue that an experimental focus on heterochromatin blocking will more likely identify novel functions related to nuclear order.

Boundary activities targeted to the UASg of KIY54 establish a nonsilenced minidomain around *ADE2* (=ON) while maintaining the flanking regions in a repressed state (*URA3* = OFF). This ON/OFF epigenetic state must stochastically be established in a fraction of cells by the targeted BAs and then propagated during growth. The number of ON/OFF cells varies, ranging from 30% for the strongest boundary proteins (Cse1p, Los1p, and Mex67p) to 5% for the weaker activities (Nup2p). This percentage number must depend on the relative boundary potential of a given protein, but also must be governed by parameters such as expression levels, protein turnover, strength of DNA and protein association, and relative cellular toxicity of the expressed protein.

Previous studies have identified in *Saccharomyces cerevisiae* certain upstream activating sequences, called UASrpg, that are associated with several ribosome protein genes and can block epigenetic repression (Bi and Broach, 1999). Likewise, it was suggested that the $tRNA^{Thr}$ gene could serve as a heterochromatin boundary element (Donze and Kamakaka, 2001). The activity of either *cis*-acting element was determined by using a similar experimental setup, where they were interposed between the silencing source and the promoter of a reporter gene. In this upstream position, these elements could block silencing either by transcription activation or heterochromatin blocking. Possibly favoring the former mechanism, it was shown that the transcriptional potential of the $tRNA^{Thr}$ is critical for its blocking activity (Donze and Kamakaka, 2001). Theoretically, it might be possible to address this question by replacing the UASg sites of KIY54 either by the UASrpg and $tRNA^{Thr}$ elements and to determine the resulting expression states.

The NPC Connection

Current studies indicate that all transport complexes generally dock to the inner NPC basket during translocation in either direction (Damelin and Silver, 2000). This interaction with the NPC basket appears to be the common molecular denominator that explains how the structurally different transportins can mediate a BA. The strongest evidence for this molecular scheme is that the BA of all transportins, but not that of BEAF, depends on Nup2p. Nup2p is predominantly associated with the inner NPC basket where it serves as a receptor for Cse1p and probably other proteins. In a Δ*nup2* strain, Cse1p is no longer NPC bound but is predominantly distributed throughout the nucleoplasm (Hood et al., 2000). This redistribution and loss of NPC interaction in a Δ*nup2* strain most likely leads to the loss of the BA of Cse1p. Perfectly in line with this interpretation is the finding that association with the nuclear rim and boundary activity of the GFP-tagged Cse1p comap precisely to the same C-terminal fragment (Figure 3).

To our knowledge, there is no clear evidence that Los1p and Mex67p are displaced from the nuclear rim in a Δ*nup2* strain and that RNA export is affected. Current thinking suggests that transport complexes ratchet along various NPC sites during translocation, probably following an interaction affinity gradient (Ribbeck and Gorlich, 2001). Although the BA of all transportins is dependent on Nup2p, this protein could be a facultative ratchet site for translocation of some complexes (e.g., those required for RNA export), but it might be the only NPC anchor that can block propagation of heterochromatin. Indeed, targeting Nsp1p and Nup1p, which functionally overlap with Nup2p, to the *HML* reporter did not result in a significant BA (data not shown).

Foremost, the NPC tethering model is supported by the observation that Nup2p itself can directly mediate a BA if it is targeted to the UASg sites of KIY54. Consequently, heterochromatin blocking can occur by direct or indirect interactions, a conclusion supported by our observation that every member of the Cse1p export complex (Cse1p, Srp1p, or Gsp2p) can exert a BA if fused to the Gbd DNA binding domain. Hence, it is unlikely that the BA is an intrinsic property of these transportins, but rather that it is exerted by NPC tethering involving Nup2p.

Our immunofluorescence, in vivo time-lapse, and ChIP studies corroborate the anchoring hypothesis. They demonstrate that the C-terminal domain of $Cse1p^{474–960}$, which encompasses its BA, can mediate the physical tethering of different genomic loci, including *HML*, to the NPC (Figures 5, 6, and 7). These morphological and biochemical observations are important since they provide a mechanistic link between the genetic results and nuclear organization.

Although the data presented strongly support a NPC tethering model, it is important to mention that we cannot completely rule out that epigenetic switching may occur when Nup2p is detached from the pore. This formal possibility arises since recent evidence demonstrated that Nup2p, although predominantly associated with the NPC, is not a typical structural static NPC component but displays some dynamic properties, in that it is also found in the nucleoplasm and cytoplasm (Dilworth et al., 2001). Consistent with the dynamic behavior of Nup2p are the time-lapse studies. We observed that LacO foci tethered to the NPC as mediated by the C-terminal domain of Cse1p occasionally visit the nuclear lumen and perhaps even the cytoplasm. We consider this latter possibility intriguing. Although a translocation to the cytoplasm of Nup2p and of its associated

exportins is expected, it is surprising that perhaps chromatin sections (the LacO array) can be dragged through the NPC channel.

A recent important study by Corces' laboratory has suggested that the boundary function of the suppressor of Hairy-wing may be exerted by tethering (Gerasimova et al., 2000). Immunofluorescence studies indicated that a DNA sequence normally located inside the nucleus appears to move to the periphery when the gypsy insulator was placed within the sequence. It will be of great interest to dissect the molecular details of this relocalization and to ask whether it implicates interactions with the NPC.

It is important to point out that NPC anchoring is only one of the mechanisms whereby boundary functions can be exerted. By way of example, the BA of BEAF is not dependent on *NUP2*, and GFP-tagging studies showed that this protein is not associated with the NPC in yeast (K. Ishii and U.K. Laemmli, submitted).

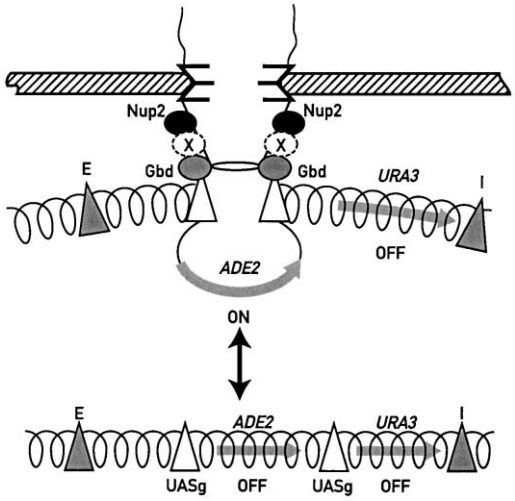

Figure 8. Specific NPC Anchoring Can Establish a Protected Chromatin Domain

This cartoon summarizes the data showing that specific tethering is one of the mechanisms whereby chromatin boundaries can be established. Note that BEAF operates differently and is not NPC bound. Two epigenetic states of the *HML* reporter are shown. The bottom of this figure shows the *HML* reporter of KIY54 in its silenced epigenetic OFF/OFF state where *ADE2* and *URA3* are not transcribed. Coils represent silenced heterochromatin. The filled triangles E and I represent the silencer elements, and the open triangles represent the UASg binding sites for the Gal4-DNA binding domain (Gbd). The top of this figure depicts tethering of the UASg sites to the NPC receptor Nup2p with the chimeric transportins (Gbd-X). This specific interaction blocks propagation of heterochromatin into the domain around *ADE2*. This chromatin boundary activity can also be obtained by targeting Nup2p directly to the UASg sites. Hence, genuine boundary activities establish a euchromatic (nonsilenced) domain around *ADE2* (=ON) while maintaining the flanking regions in a repressed state (*URA3* = OFF). Importantly, since Nup2p is a dynamic NPC component, we emphatically do not rule out that heterochromatin blocking may occur when Nup2p is *HML* bound but detached from the NPC.

Distinct Peripheral Nuclear Compartments

How does Nup2p/NPC tethering block propagation of heterochromatin at the molecular level? Somehow, this interaction has to ensure that the *ADE2* minidomain of KIY54 is insulated from silencing (Figure 8). This molecular process is limited to the minidomain of about 2.4 kb by the straddling boundary proteins. Heterochromatin blocking might be achieved by "energetic unlinking" of the minidomain from its flanking sections so that association and maintenance of the Sir complex is disfavored. Energetic unlinking could occur by active or passive processes such as physical anchoring. Concerning the latter, association of the Sir silencing complex is known to alter the extent of DNA supercoiling (Bi and Broach, 1997; Cheng et al., 1998). If the linking-number change associated with silencing cannot physically propagate into the insulated domain, then this section is energetically unlinked and heterochromatin formation is disfavored. The minidomain may operationally behave like a topological independent domain, like a loop, akin to DNA rings that were separated from the silencers. Previous experiments demonstrated that DNA rings excised from a silenced region and bearing no silencers reactivate (Cheng and Gartenberg, 2000).

This passive anchoring model, however, is not entirely satisfactory since if physical restriction were the only mechanism whereby the BA function is obtained, then genetic screening should have identified many other anchors. As a possibility, we consider a combination model where tethering to Nup2p initiates an active process of chromatin remodeling.

In a more general scenario, association with the NPC compartment could possibly favor the formation of accessible chromatin or the epigenetic switching to this state. That is, the NPC compartment could simply be generally rich in chromatin "activating" functions (e.g., histone acetyl-transferases) or be poor in silencing factors. Although the experiments presented here favor an anchoring model, they provide no molecular insight into other possibilities. To address these mechanistic questions, it will be of interest to screen for yeast mutants that have lost the ability to mediate the Nup2p-dependent epigenetic switching.

It has often been proposed that the nuclear periphery of budding yeast represents a compartment associated with silencing. This discussion stems from the notion that the silenced telomeres of yeast often localize to the nuclear periphery and are clustered into foci (reviewed in Cockell and Gasser, 1999). The observation that tethering of genes to the nuclear membrane (as opposed to the NPC) leads to silencing (Andrulis et al., 1998) supports this view. It is noteworthy to point out that the targeting of some constructs, which mediate membrane binding (Gbd-Yip1$^{1-248}$ and Gbd-Yif1$^{55-314}$), to the UASg sites of KIY54 did not reveal a BA function nor increase silencing of the *HML* locus (data not shown).

The Mlp1p and Mlp2p proteins (homologous to the mammalian TPR proteins), which appear to form long extensions from the nuclear NPC basket into the nuclear lumen (Strambio-de-Castillia et al., 1999), are thought to be involved in establishing the repressive perinuclear compartment of yeast (Galy et al., 2000). We determined that the *MLP* genes are not involved in the BA of the transportins and BEAF, as their BA was not affected in a strain harboring a Δ*mlp1*/Δ*mlp2* double deletion (data not shown).

In summary, the data presented in this study indicate that the nuclear periphery is not generally repressive but is differentiated into at least two compartments, the silencing membrane-telomere compartment and the molecularly defined, nonrepressive (desilencing?) NPC space.

Physiology

We currently do not know whether genomic loci physically interact with the NPC as part of normal cell physiology. Given the specificity and efficiency of the epigenetic effect described here and the dynamic behavior of genomic loci that sample a large volume of the nucleus (Marshall et al., 1997; Heun et al., 2001), it would be surprising if such biological potentials were evolutionarily ignored and if the observations made here would only be reflective of a molecular example of heterochromatin blocking. Time-lapse studies showed that moving genomic loci frequently ricochet off the NPC periphery. Do such loci, while briefly anchored to NPC, obtain epigenetic jolts toward activation? Do visiting loci also acquire more efficiently imported NLS cargo by direct transfer (not diffusion)? Does this process favor the formation of accessible chromatin? Are the NPCs not only entry gates, but also signal stations that convey cellular physiology to the nucleus?

Although results of our experiments raise many unanswered questions about nuclear function, they finally provide strong evidence for a molecular scenario whereby nuclear anchoring can dramatically alter the epigenetic behavior of a gene. Perhaps further studies using a combination of genetics, structural, and biochemical tools will provide the inroad toward a better elucidation of the relationship of nuclear structure to gene function and epigenetic phenomena.

Experimental Procedures

Plasmids and Yeast Strains

The Gbd fusion yeast genomic library used in this study has been described by Andrulis et al. (1998). Library-derived pGBC11-Cse1$^{474–960}$ was self-ligated between ClaI and PstI sites to create Gbd-Cse1$^{474–912}$. Gbd-Cse1$^{1–960}$, Gbd-Cse1$^{659–960}$, Gbd-Nup2$^{1–720}$, and Gbd-Srp1$^{1–542}$ were made by PCR amplification of corresponding genomic DNA fragments, followed by insertion into pGBC11 or its derivative pGBC12 to allow their in-frame expression with Gbd (K. Ishii and U.K. Laemmli, submitted). The AatII-PstI fragment from pGBC11-Cse1$^{474–960}$ was used to create Gbd-Cse1$^{585–960}$ in pGBC11. Gbd-Nup2$^{1–362}$ and Gbd-Nup2$^{217–720}$ were made by removing NsiI-SalI and NcoI-SwaI fragments from pGBC12-Nup2$^{1–720}$. Gbd-Los1$^{103–884}$ was made by excision of a PstI fragment from pGBC11-Los1$^{103–1100}$; the excised fragment was inserted into pGBC11 to create Gbd-Los1$^{885–1100}$. Replacing the NsiI-DraIII fragment of pGBC11-Los1$^{103–1100}$ with the PstI-DraIII fragment from pGBC11 created Gbd-Los1$^{787–1100}$. For Gbd-Cse1$^{474–960}$-GFP, a SalI site was created by PCR just before the stop codon of the corresponding Cse1 fragment, and the GFP fragment from pFA6a-GFP-kan^R (Longtine et al., 1998) was inserted in the correct reading frame. ClaI-PstI fragment from this construct was used to replace the C terminus of Gbd-Cse1$^{659–960}$ to create Gbd-Cse1$^{659–960}$-GFP. Gbd-Cse1$^{474–912}$-GFP was obtained by the same manipulation of Gbd-Cse1$^{474–912}$.

Yeast strains used in this study are derivatives of W303-1a (a *ade2-1 ura3-1 trp1-1 leu2-3, 112 his3-11, 15 can1-100*). Manipulation of yeast and spotting assays with KIY54 are as described in K. Ishii and U.K. Laemmli (submitted). *nup2*-deleted strains YGA1 and YGA2 were made by PCR-mediated one-step gene disruption in KIY54 using *his5*$^+$ and kan^R, respectively (Longtine et al., 1998). GFP-Cse1$^{474–960}$-LacI strains were made by inserting a PCR-amplified Cse1$^{474–960}$ fragment into the SmaI site of pAFS135 (Heun et al., 2001), followed by NheI digestion and genomic integration at the *HIS3* locus of strains carrying a LacO array (256 multimerized repeats) at either ARS908 or ARS1413 (Heun et al., 2001). The *nup2* deletion was then introduced by crossing with YGA2. Strain CLY1 is a derivative of KIY54 whose *SIR3* was tagged with 13 myc epitopes (Longtine et al., 1998). In addition, its *ade2-1* and *ura3-1* alleles were exchanged with *LEU2* and *his5*$^+$, respectively, by PCR-mediated gene replacements.

Library Screen

The screen for boundary activity in KIY54 with yeast Gbd-hybrid library was repeated three times in this study. Typically, $\sim 1.5 \times 10^7$ transformants were screened by direct plating on SC −Trp −Ade +FOA, and plasmids recovered from individual colonies on the plates were further verified for the activity by retransforming them into KIY54. So far, 38 different plasmids have been selected. The transportins obtained in the screen are the following: Cse1p (aa 474–960, 5–891), Los1p (aa 103–1100, 271–1100, 446–1100), Mex67p (aa 248–599), Sxm1p (aa 2–881), and Gsp2p (aa 8–214).

Chromatin Immunoprecipitation with MAb414

A solution of formaldehyde-fixed chromatin was prepared as described in Kuras and Struhl (1999). About 50% of Nup2p and other MAb414-reacting NPC proteins were solubilized into the chromatin solution after sonication. The DNA in the soluble fraction was used as total and had an average size of 500 base pairs (bp) and a range between 200 and 1000 bp. Immunoprecipitation was performed by applying 6.25 μg/ml of MAb414 precoupled to anti-mouse IgG-conjugated paramagnetic beads (Dynabeads M-450, Dynal) to 0.8 ml of the chromatin solution prepared in FA-lysis buffer (50 mM HEPES-KOH [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) supplemented with 1% Triton-X 100 for 4 hr at 4°C. Note that the efficiency of NPC protein recovery in this procedure was 10%–20%. Elution of immunoprecipitated material and preparation of DNA were according to standard procedures (Kuras and Struhl, 1999). DNA concentration was determined by Picogreen fluorescence (Molecular Probes). Enrichment of immuno-isolated DNA from the LacO-containing strain was examined by quantitative Southern blot (Käs and Laemmli, 1992). To detect the association of *HML* in strain CLY1, real-time PCR was performed using the ABI Prism 7700 Sequence Detection System according to the Taqman Universal PCR master mix protocol (PE Biosystems). Approximately 2 ng of DNA was used for each real-time PCR, and the resulting enrichment and the standard deviation were calculated from the triplicate reactions with at least two different ChIP DNA preparations. The following oligonucleotides were used as the Taqman probes CAAGTCAGAGTCTGATCCCATGATGATTCCA (*HML-ADE2*) and TTGCAATTCGCCCGCGTCTG (*PRD1*), and the PCR primers TGGAAGCAATGGTCAAACCAT/CACATGCGGCAGACATTACC (*HML-ADE2*) and TCTATCTCTGCGTCACGACATCTT/TGCCTTCG ATTGAACATCCTG (*PRD1*).

Immunofluorescence Microscopy and Live Imaging

Exponentially growing cultures (40 ml) were fixed with 2% or 4% (final) freshly prepared paraformaldehyde for 1 hr at room temperature (RT), and the reaction was quenched by the addition of 125 mM (final) of glycine (5 min at RT). The cultures were then washed by centrifugation three times with YPD (5 ml each), and the final pellet was taken up in 2 ml of KED (0.1 M K/EDTA [pH 8.0], 10 mM DTT) for 30 min at RT. Following centrifugation, cells were taken up in 2 ml of YPD containing 20% (w/v, final) of sorbitol (YPDs). Spheroplasting was initiated by the addition of zymolyase (final: 0.5 mg/ml) and a titrated amount of lyticase to obtain spheroplasts in about 20 min by incubation (30°C) and gentle agitation. Spheroplasts were then washed by centrifugation with YPDs (3 times, 10 ml each), and the final pellet was resuspended in 1 ml of YPDs diluted to obtain a final concentration of 50% glycerol for storage at −20°C. For staining, 30 μl of spheroplasts were diluted into 0.5 ml of HEN-TS (10 mM HEPES [pH 7.4], 100 mM NaCl, 1 mM EDTA, 0.15% Triton X-100, and 3.4% sucrose) and after 10 min (RT) spun onto polylysine-coated coverslips (Boy de la Tour and Laemmli, 1988). Coverslips

were blocked 20 min at RT with normal goat serum 3% (final) diluted into HEN (lacks Triton and sucrose) and stained by standard procedures with the anti-NPC monoclonal antibody MAb414 (Covance) and anti-GFP (Roche) antibodies, respectively. The former antibody (MAb414) was localized by a secondary goat anti-mouse antiserum (Texas red-labeled) and the latter (anti-GFP) by a goat anti-rabbit antiserum (fluorescein-labeled). Samples were finally mounted in PPDI (5 mM HEPES [pH 7.7], 100 mM NaCl, 20 mM KCl, 1 mM EGTA-KOH [pH 8.0], 10 mM MgSO4, 2 mM CaCl2, 78% glycerol, and 1 mg/ml of paraphenylene diamine) supplemented with DAPI at 1 μg/ml). Live imaging of cells was carried out essentially as described by Heun et al. (2001). Images were recorded with a wide-field, deconvolution-type imaging system from DeltaVision.

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